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(57) Abstract

Processes for preparaing biodegradable collagen-based matrices in sponge or sheet form wherein in one embodiment a collagen-based material including a collagen selected from the group consisting of types I, II and III collagens is freeze dried to form a collagen-based sponge which is contacted with a crosslinking agent selected from the group consisting of a carbodiimide or a succinimidyl active ester to form an intermediate collagen-based matrix which is subsequently subjected to conditions of severe dehydration to form the collagen-based matrix in sponge or sheet form. In another embodiment, a collagen-based sponge or sheet is first subjected to conditions of severe dehydration followed by contacting the thus formed intermediate collagen-based matrix with a carbodiimide crosslinking compound to form the collagen-based matrix in sponge or sheet form. In still another embodiment of the present invention the cross-linking agent is admixed with the collagen-based material prior to formation of the collagen-based sponge or sheet followed by processing s steps of severe dehydration. In a particularly preferred form of the invention, a carrier compound is incorporated during processing to form a collagen-based matrix in sponge or sheet form impregnated with a carrier compound wherein the carrier compound is selected from the group consisting of types IV and V collagen, fibronecting, laminin, hyaluronate, proteoglycan, epidermal growth factor, platelet derived growth factor, angiogenesis factor, antibiotic, antifungal agent, spermacidal agent, enzyme and enzyme inhibitor.

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BIODEGRADABLE MATRIX AND METHODS FOR PRODUCING SAME

Field of the Invention

This invention relates to a biodegradable matrix, and more particularly to a multifunctional collagen-based matrix and/or carrier system thereof.

Background of the Invention

Delivery of drugs, growth factors, hormones, peptides and glycopeptides to external wounds has classically occurred by direct topical application and application to internal wounds by injection into the blood or by absorption into the blood through the digestive system. Controlled release of these agents has been achieved by encapsulation either in bulk or at a microscopic level using synthetic polymers, such as silicone, and natural polymers, such as gelatin and cellulose. The release rate can be controlled for periods of up to a year by proper choice of the polymeric system used to control the diffusion rate (Langer, R.S. and Peppas, N.A., Biomaterials 2,201,1981). Natural polymers, such as gelatin and cellulose slowly dissolve in a matter of minutes to hours while silicone remains intact for periods of months to years. Biodegradable polymers offer an advantage for controlled release to internal wounds since only a single surgical procedure is necessary.

Collagen is a biodegradable polymer found in animals and in man. It has been used as a plasma expander, vehicle for drug delivery, vitreous body replacement, hemostatic agent, suture material, corneal replacement, hemodialysis membrane, wound dressing and artificial skin, hernia patch, vessel prosthesis, vaginal contraceptive, and injectable agent for tissue augmentation (Chvapil et al., Int. Review of

Connective Tissue Research 6, 1, 1973; Chvapil, in Biology of Collagen edited by A. Viidik and J. Vuust, Academic Press, chapter 22, 1980). In most of these applications the collagen is reconstituted and crosslinked into an insoluble form.

There is described in Yannas et al., (U.S. patent 4,060,081), the use of collagen and mucopolysaccarides as synthetic skin. Such material is crosslinked using glutaraldehyde, a bifunctional crosslinking agent, which reacts with free amines. One major drawback to using crosslinked collagen has been the adverse biological effects of free glutaraldehyde, a common agent used to crosslink and insolubilize collagen in many applications. Leaching of glutaraldehyde from crosslinked collagens has been shown to be cytotoxic to cells, specifically fibroblasts (Speer et al., J. Biomedical Materials Research 14,753,1980; Cooke et al., British J. Exp. Path. 64,172,1983). Recent evidence suggests that glutaraldehyde polymers and not monomeric glutaraldehyde form crosslinks between collagen molecules; these crosslinks can then rearrange to release free glutaraldehyde and glutaraldehyde polymers (Cheung, D.T. and Nimni, M.D., Connective Tissue Research 10,187-217,1982).

Objects of the Present Invention

An object of the present invention is to provide a novel biodegradable matrix.

Another object of the present invention is to provide a novel collagen-based matrix.

Still another object of the present invention is to provide a novel collagen-based matrix in sponge or sheet form.

Yet another object of the present invention is to provide a novel biodegradable matrix impregnated with a carrier compound.

A further object of the present invention is to provide a novel biodegradable collagen-based matrix impregnated with a carrier compound.

Yet still another object of the present invention is to provide a novel biodegradable collagen-based matrix impregnated with a carrier compound in sponge or sheet form.

A still further object of the present invention is to provide a novel biodegradable collagen-based matrix impregnated with a carrier compound and is non-toxic and capable of promoting cell growth.

Yet another object of the present invention is to provide a novel biodegradable collagen-based matrix impregnated with a carrier compound for controlled release of drugs.

Yet still another object of the present invention is to provide a novel biodegradable collagen-based matrix impregnated with a carrier compound for topical application to external wounds.

Another object of the present invention is to provide a novel biodegradable collagen-based matrix impregnated with a carrier compound for application to internal wounds.

Summary of the Invention

These and other objects of the present invention are achieved in one embodiment by forming a sponge or sheet of a collagen-based material including a collagen selected from the group consisting of types I, II and III collagens which sponge or sheet is contacted with a cross-linking agent selected from the group consisting of a carbodiimide or a succinimidyl active ester to form an intermediate collagen-based matrix which is subsequently subjected to conditions of severe dehydration to form a collagen-based matrix in sponge or sheet form. In another embodiment, the sponge or sheet of the collagen-based material is first subjected to conditions of severe dehydration followed by contacting the thus formed intermediate collagen-based matrix with a carbodiimide crosslinking compound to form the collagen-based matrix in sponge or sheet form. In still another embodiment of the present invention the cross-linking agent is admixed with the collagen-based material prior to formation of the intermediate collagen-based sponge or sheet followed by processing steps of severe dehydration. In a

particularly preferred form of the invention, a carrier compound is incorporated during processing to form a collagen-based matrix in sponge or sheet form impregnated with a carrier compound wherein the carrier compound is selected from the group consisting of types IV and V collagen, fibronectin, laminin, hyaluronate, proteoglycan, epidermal growth factor, platelet derived growth factor, angiogenesis factor, antibiotic, antifungal agent, spermacidal agent, enzyme and enzyme inhibitor.

Detailed Description of the Invention

The collagen-based carrier systems of the present invention are based on the use as a starting material of a soluble or insoluble collagen selected from the group consisting of types I, II and III collagens and mixtures therof.

Soluble collagens of the types I, II and III collagen are prepared by limited enzymatic digestion of tissue enriched in such collagen types and are formed into collagen-based solution (i.e. a soluble collagen dissolved in a suitable solvent, such as dilute hydrochloric acid, dilute acetic acid or the like).

Insoluble collagens are derived from the following typical sources: type I collagen; bovine, chicken and fish skin, bovine and chicken tendons and bovine and chicken bones including fetal tissues; type II collagen: bovine articular cartilage, nasal septum, sternal cartilage; and type III collagen; bovine and human aorta and skin.

In one embodiment of the present invention, a collagen-based solution or an insoluble collagen dispersed and swollen in a suitable liquid media (e.g. dilute hydrochloric acid, dilute acetic acid or the like) is subjected to a temperature of between about 0°C. to -100°C. to thereby solidify the collagen-based material. Thereafter, the solidified collagen-based material is subjected to a vacuum of less than about 50 millitorr at a temperature of from about 22°C. to -100°C. to form a collagen-based sponge to be further processed, as hereinafter more clearly described. Generally, a weight ratio of soluble or

insoluble collagen to solvent or dispersion agent, respectively, of from 1 to 10,000 or from 1 to 15 is used to form the collagen-based solution or dispersion.

In another embodiment of the present invention such a collagen-based solution on a collagen-based dispersion is dried into sheet form prior to further processing as more fully hereinafter described. Drying is effected at temperatures of from 4°C. to 40°C for a period of time of from 2 to 48 hours.

In one embodiment of the present invention to form a collagen-based matrix, a collagen-based sponge or sheet is first contacted with a crosslinking agent selected from the group consisting of a carbodiimide or N-hydroxysuccinimide derived active esters (succinmidyl active ester) followed by severe dehydration to form the collagen-based matrix. Examples of the carbodiimides include cyanamide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride. Examples of bifunctional succinimidyl active esters including bifunctional N-hydroxy-succinimide, 3,3^1-dithio (sulfosuccinimidyl proprionate and bis (sulfo-succinimidyl) suberate. When using a carbodiimide crosslinking agent, the collagen-based sponge or sheet is immersed in a carbodiimide solution at a concentration of from about 0.1 to 10% (W/V) maintained at a temperature of from about 2 to 40°C. and at a pH of between 2 to 11 for a period of time of from about 2 to 96 hours. When using a succinimidyl active ester crosslinking agent, the collagen-based sponge or sheet is immersed in a solution thereof at a concentration of from about 0.1 to about 15.0%(W/V) maintained at a temperature of from about 2 to 40°C. for a period of time of from about 2 to 96 hours. The collagen-based sponge or sheet is placed in a solution containing 0.1 to about 15% (W/V) of N-hydroxysuccinimide and carbodiimide at a pH between 2 to 11 for a period of time between 2 to 96 hours at a temperature of from about 2°C. to 40°C. The thus treated intermediate collagen-based matrix is exhaustively washed to remove excess crosslinking agent.

Severe dehydration of the intermediate collagen-based involves conditions of temperature of from 50°C. to 200°C. at a vacuum of 50 millitorr or less for a period of time of from 2 to 96 hours to thereby form the collagen-based matrix. Prior to severe dehydration, the intermediate collagen-based matrix in sponge form is preferably solidified at a temperature of between about 0°C. to -100°C. and thereafter subjected to a vacuum of at least 50 millitorr at a temperature of between about 22°C. and -100°C.

In another embodiment of the present invention to form the collagen-based matrix when using a carbodiimide crosslinking agent, severe dehydration may be effected to form an intermediate collagen-based matrix prior to contracting such an intermediate collagen-based matrix with such a crosslinking agent.

In another embodiment of the present invention to form the collagen-based matrix, the cross-linking agent is premixed with the collagen-based material prior to drying or initiating of freeze drying processing steps.

The collagen-based matrix prepared in accordance with the present invention may be described as a "coral-like" or "scaffold" structure having interstices of a pore size of from 3 to 100 μm., and of a molecular weight of from 10 X 10^6 to in excess of 50 X 10^6 with a molecular weight between crosslinks from 1,000 to 100,000 via the formation of covalent bonds.

Another embodiment of the present invention is the incorporation of a carrier compound into the collagen-based matrix. Such a carrier compound is selected from the group consisting of collagen types IV and V, fibronectin, laminin, hyaluronate, proteoglycans, epidermal growth factor, platelet derived growth factor, angiogenesis factor, antibiotic, antifungal agent, spermacidal agent, hormone, enzyme and enzyme inhibitor.

Generally, the carrier compound may be introduced at any time during processing of the collagen-based material to the collagen-based

matrix in sponge or sheet form. Preferably, a carrier compound is incorporated prior to solidifying of the soluble or insoluble collagen in forming the intermediate collagen-based matrix, or prior to solidifying of the intermediate collagen-based matrix prior to severe dehydration. The carrier materials are added in an amount of from 1 to 30% (w/w) based on the weight of soluble or insoluble collagen. Generally, connective tissue factors, such as fibronectin, types IV and V collagens; laminin, glycosaminoglycans and hyaluronate are incorporated during initial processing steps in forming the collagen-based sponge, or after crosslinking of the intermediate collagen-based matrix. It is preferable to incorporate fibronectin and hyaluronate during forming of the intermediate collagen-based matrix.

There are many sources for the diverse carrier compounds for incorporaton into the collagen-based matrix constituting one embodiment of the present invention. Type IV and V collagens are found associated with basement membranes and smooth muscle cells in tissues, respectively. Typical sources of Type IV include the EHS mouse sarcoma tumor, bovine and human placenta, lens capsule and kidney; sources of Type V collagen include placental membranes, ocular tissues and fetal skin (see for example, Trelstad, In Immunochemistry of the Extracellular Matrix, Vol. 1 edited by H. Furthmayr, CRC Press, chapter 2, 1982).

Typical sources of proteoglycans include bovine and human cartilage and synovial fluid, nasal septum and sternal cartilage, and skin. Typical sources for glycoproteins include EHS tumor, bovine and human kidneys, cartilage, bone and placenta as well as bovine and human blood. Typical sources of hyaluronate include rooster comb and bovine vitreous.

Preferred carrier compounds for the collagen-based matrix include fibronectin, laminin, type IV collagen and complexes of hyaluronate and proteogylcans. A value of the swelling ratio of between 2.5 to 5 is required for a collagen-based matrix which comes into contact with

open wounds, while a swelling ratio of between 2.5 to 10 are useful for a collagen-based matrix including carrier compounds for subcutaneous implantation. The swelling ratio is defined as the volume of water absorbed per unit volume of collagen-based matrix.

In the case of internal wounds and short-term drug release, collagen-based matrix including a carrier compound in the form of a sheet or a tube is placed in direct contact with tissue.

The biodegradation rate and the release rate can be controlled by variation of the crosslink density. For applications where long term release is desired, a nonporous diffusion layer of a biodegradable polymer, such as poly-3-(hydroxybutyrate) is applied to each side of the collagen-based matrix. For materials to be used on full thickness wounds, a diffusion control layer, such as described in the aforementioned U.S. Letters Patent to Yannas, is applied to prevent diffusion of water or other small volatile molecules from the collagen-based matrix; the diffusion control layer must adhere tightly to the collagen-based matrix layer. The combination of collagen-based matrix layer and diffusion control layer must be strong enough to be sutured and have a tensile strength of at least 100 psi and be able to deform at least 10% before failing. Synthetic non-biodegradable polymers, such as silicone polymers (Silastic Medical Grade Adhesive) or biodegradable polymers, such as polylactic acid, polyglycolic acid, poly-3-(hydroxybutyrate) and copolymers of these materials can be used as the diffusion control layer.

Silicone polymers are preferred materials to control the rate of diffusion of small molecules. Curing of the adhesive in the diffusion control layer can occur wthout wetting the carrier compound in the collagen-based matrix by dessication at room temperature. A film 0.5 to 1.5 mm in thickness is applied to the matrix layer and is allowed to cure at room temperature for at least 2 hours using a vacuum of 14 in. of Hg.

The thickness of the collagen-based matrix can be varied from 1 to several hundred mm. For full thickness wounds, a thickness of 2 to 3 mm. is desired and enables close contract between the carrier compound in the collagen-based matrix and the wound bed.

When implanted subcutaneously or directly on full thickness dermal wounds, any collagen-based matrix of the present invention does not stimulate the inflammatory process. In addition, chronic implantation results in fibroblast and endothelial cell migration into the collagen-based matrix. The degradation and resorption rate of collagen-based matrices are as assayed in vitro by determination of the time that 1 mg. of the material resists solubilization by 100 units of bacterial collagenase. One unit of collagenase liberates amino acids in collagen equivalent to 1.0 micro mole of L-leucine in 5 hours at 37°C. A combination of succinimidyl active ester formation and severe dehydration or carbodiimide treatment and severe dehydration increases the collagenase resistance time significantly over that observed by any of the procedures heretofore used. These studies indicate that the crosslinking methods of the present invention result in resistance to collagenase degradation and show no stimulation of inflammation.

EXAMPLES OF THE INVENTION

The following examples are illustrative of conditions for the process of the present invention and it is to be understood that the scope of the invention is not to be limited thereby.

EXAMPLE 1

Preparation of Soluble and Insoluble Collagens

The collagen used is prepared from calf hides after the grain layer is separated from the corium or dermis. The corium is cut into pieces, washed, swollen, freeze dried and stored at -20°C.

Soluble collagen is obtained by placing 250 gr. of the freeze dried insoluble collagen in 1.5 liters of HCl at a pH of 2.5 containing 1.5 gr. of crystalline pepsin (Sigma Chemical Company). The mixture is stirred overnight at 4°C. and then filtered through cheese cloth,

Whatman filter paper #4 followed by Whatman #42, Gelman 5 μm and 1.2 μm filters and finally through Millipore 0.65 μm and 0.45 μm filters. The soluble fraction obtained after sequential filtration contains types I, III and V collagens. The soluble collagen fraction is dialyzed against a 1.0 M NaCl solution containing 50 mM Tris adjusted to pH 7.5 and enough solid NaCl is added to raise the molarity of the solution to 1.7. The precipitate at 1.7 M NaCl is type III collagen and is collected by centrifugation at 10,000 x g for 60 minutes and then dialyzed versus 0.005 M acetic acid, freeze dried and stored at -20°C. To the supernatant is added additional NaCl to raise the molarity to 2.5 M and the precipitate (type I collagen) is pelleted by centrifugation, dialyzed versus 0.005 M acetic acid, freeze dried and stored at -20°C. The remaining supernatant contains type V collagen and is dialyzed against 0.005 M acetic acid, freeze dried and stored at -20°C.

Insoluble collagen solutions are obtained by dispersing freeze dried corium ground using a Wiley Mill in HCl at pH of 2.5. The swollen collagen is then dialyzed against 0.005 M acetic acid and freeze dried. If mature bovine hide is the source of the corium, the insoluble collagen produced is typically type I.

Soluble type IV collagen is extracted from the mouse EHS sarcoma tumor after washing the tumor in cold distilled water 3 times by spin filtration at 10,000 x g for 30 minutes. The pellet (0.500 gr) is homogenized in 0.5 liters of 0.5 M acetic acid adjusted to pH 2.5 with HCl and 0.5 gr of pepsin is added. The homogenate is allowed to mix at 4°C. for 24 to 48 hours, filtered through cheese cloth, spun at 10,000 x g for 30 minutes. The pellet is re-suspended in 0.5 M NaCl containing 50 mM Tris, NaCl is added until a final concentration of 4.5 M is obtained, centrifuged at 10,000 x g for 30 minutes and the pellet is dialyzed against 0.1 N acetic acid 3 times and freeze dried.

EXAMPLE 2

Preparation of Bovine Serum Fibronectin

Freshly drawn bovine blood (80 ml) is collected in a polypropylene tube containing 20 ml. of 5% trisodium citrate and 0.1 mM phenylmethysulfonyl fluoride. The blood is centrifuged at 300 x g for 10 minutes and the plasma is then separated from the cell layer and further centrifuged at 30,000 x g at a temperature of 15°C. for 30 minutes. To the plasma is then added an additional 1 mM of phenylmethylsulfonyl fluoride and then it is poured through a gelatin-sepharose column. The column is washed with a phosphate buffer solution (0.182 M phosphate and 0.145 M NaCl), 2 column volumes 1M NaCl in a phosphate buffer, 2 column volumes of phosphate buffer solution, 2 column volumes of 4 M urea in a phosphate buffer. The optical density of column fractions is monitored at a wavelength of 280 nm with the bulk of the fibronectin normally present in the fractions containing 4 M urea. Fibronectin containing fractions having optical densities greater than 0.1 are dialyzed against 0.180 M phosphate buffer containing 0.145 M Nacl (pH 7.2). The sample is then dialyzed against 0.05 M Tris-HCl containing 4.5 M urea (pH 7.2) and applied to a DEAE (diethylaminoethyl cellulose) ion exchange column at room temperature. The column is eluted with a 0 to 0.3 M NaCl linear gradient in a 0.05 M Tris-HCl buffer solution (pH 7.2) containing 4.5 M urea. The eluted fibronectin is dialyzed against 0.180 M phosphate containing 0.145 M NaCl and 1.0 M urea and frozen at -20°C.

EXAMPLE 3

Preparation of Laminin

Laminin is prepared for lathrytic EHS tumors grown in C57/6J mice. The tumors are dissected and homogenized in 3.4 M NaCl containing 50 mM Tris-HCl adjusted to pH 7.4 and protease inhibitors. The homogenate is centrifuged at 16,000 x g for 60 minutes the pellet is collected and resuspended in 3.4 M NaCl. Following centrifugation

at 16,000 x g for 60 minutes a second time, the homogenate is resuspended in 0.5 M NaCl containing 50 mM Tris-HCl pH 7.4 and protease inhibitors stirred for 18-24 hours at 4°C. followed by centrifugation at 16,000 x g for 60 min. The supernatant is brought to 3.5 M by addition of solid NaCl, stirred overnight at 4°C. and the precipitate is collected by centrifugation. Laminin is further purified by redissolving in 0.5 M NaCl containing 50 mM Tris-Hcl pH 7.4 followed by centrifugation at 10,000 x g for 60 minutes the supernatant is dialyzed against 2 M urea containing 2.5 NaCl and 50 mM Tris-HCl pH 8.6 and chromatographed over a 2.5 x 2.5 cm. column containing DEAE cellulose equilibrated with the same buffer at 4°C. The unbound fraction is dialyzed against 2 M urea containing 50 mM Tris-HCl pH 8.6 and rechromatographed on the DEAE column equilibrated with the same buffer. The unbound fraction is concentrated by vacuum dialysis and chromatographed on Sephacryl S-300 equilibrated with 1.0 M $CaCl_2$, 50 mM Tris-HCl pH 7.5 at 22°C. the void volume is collected and dialyzed against 0.4 M NaCl, 50 mM Tris-Hcl pH 7.4 and stored at 4°C. Laminin is resolubilized in 0.1 M ammonium hydroxide pH 11.0 and then the pH is adjusted to 7.2.

EXAMPLE 4

Preparation of Collagen-based Sponges and Sheets

Soluble or insoluble collagen (1.2 gr.) is added to 120 ml. of a dilute HCl solution of pH 3.0 and the mixture is ground in a Waring Blender at low speed for 1 minute and thereafter at high speed for 1 minute. The solution or dispersion is then poured into a vacuum flask and deaerated at a vacuum of 100 millitorr for 10 minutes. Collagen dispersions and solutions to be converted into sponges are cooled to 0°C. and frozen at -100°C. before freeze drying at -65°C. under a vacuum of less than 10 millitorr. Collagen dispersion or solutions to be processed into sheets were placed in a sterile hood and allowed to air dry for 24 to 48 hours at 22°C.

EXAMPLE 5

Preparation of Succinylated, Succinimidyl Ester Crosslinked Intermediate Collagen-Based Matrix

Nine grams of succinic anhydride are dissolved in 80 ml. of distilled water and mixed for 30 minutes at 37°C. After the succinic anhydride is in solution, the pH is adjusted to 7.2 and the volume brought to 100 ml. This solution is placed in a Waring Blender and 1.0 gr. of collagen is added, ground for 2 minutes, allowed to stand at 22°C. for 1 hour, placed on Whatman #4 filter paper to remove unreacted succinic anhydride and then washed with 100 ml. of distilled water.

The residue is placed in a solution of 20 ml. of phosphate buffer (0.182 M phosphate and 0.145 M NaCl) and 2 gr. of N-hydroxy-succinimide and 2 gr. of cyanamide are added. The solution pH is then adjusted to 7.2. The residue is allowed to react for 3 hours at room temperature and then washed with distilled water in a Buchner funnel under a vacuum of 14 in. of Hg. Sheets and sponges of succinylated, succinimidyl ester crosslinked intermediate collagen-based matrices are produced in accordance with the processes described in Example 4.

EXAMPLE 6

Preparation of Collagen-Based Matrix Containing Fibronectin

To 1.2 gr. soluble or insoluble collagen in 120 ml. of HCl pH 3.0 is added to 0.12 gr. of fibronectin in (2.5 mg. fibronectin/ml) 0.1 M urea containing 0.182 M phosphate and 0.145 M NaCl and the mixture is dispersed in a Waring Blender for 2 minutes. Sheets and sponges are prepared in accordance with the processes described in Example 4.

EXAMPLE 7

Sponges and Sheets Formed by Coating Type I Collagen With Laminin, Fibronectin, and Type IV Collagen

Collagen sheets and sponges formed according to Examples 4 and 6 are swollen in a 0.1 ammonium acetate pH 7.2 containing 1 to 5% laminin, fibronectin or type IV collagen. The swollen sponge or sheet is then frozen and freeze dried at -65°C. and in a vacuum less than 50 millitorr.

EXAMPLE 8

Preparation of Collagen-Based Matrix Containing Hyaluronate and Proteogycans

To 1.2 gr. of soluble and insoluble collagen in HCl pH 3.0 as added to 0.12 gr. of a complex of hyaluronate and proteoglycans (Sigma Chemical Company Grade III-P) in a HCl pH 2.0 to a final volume of 120 ml. The mixture is dispersed in a Waring Blender and either freeze dried or air dried in accordance with the processes of Example 4.

EXAMPLE 9

Preparation of Collagen-Based Matrix Containing Type IV Collagen

To 1.2 gr. of soluble or insoluble collagen type I in HCl pH 2.0 is added to 0.012 gr. of type IV collagen in a 0.1 M ammonium acetate pH 7.2 to a final volume of 120 ml. The mixture is dispersed in a Waring Blender for 2 minutes and formed into sheets or sponges in accordance with the processes of Example 4.

EXAMPLE 10

Cyanamide Crosslinking of Collagen-Based Matrix

The product of Examples 4 and 6 to 9 are crosslinked by immersion in an aqueous solution containing 1% by weight of cyanamide at pH 5.5 for a period of 24 hours at 22°C. After removal, the sponges and sheets are exhaustively washed in several changes of water over 24 hours, frozen and freeze dried at -65°C. in a vacuum of less than 50 millitorrs.

EXAMPLE 11

Crosslinking of Collagen-Based Matrix by Severe Dehydration

The products of Examples 4 and 6 to 10 are placed in a vacuum oven at room temperature and exposed to a vacuum of less than 50 millitorr. After one hour the samples are heated to 110°C and remain at this temperature for 72 hours at which time the temperature is lowered to 40°C. The samples are then removed from the vacuum oven and stored at -20°C.

EXAMPLE 12

Crosslinking of Collagen-Based Matrix Using Succinimidyl Active Ester

Two grams of succinimidyl active ester crosslinked collagen prepared according to Example 5 is placed in a Waring Blender containing 400 ml. of HCl pH 2.0 and dispersed for two minutes. This mixture is deaerated by placing in a vacuum of 300 millitorr and is then placed at room temperature in a 100% relative humidity environment for 24 hours. The material is then cooled to 0°C., frozen and freeze dried at -65°C. using a vacuum of less than 50 millitorr or air dried to make sponges or sheets, respectively.

EXAMPLE 13

Preparation of Collagen-Based Matrix Containing Protease Inhibitors

Sponges and sheets prepared according to Examples 4 to 9 are placed in 20 ml. of HCl at pH 2.0 containing 25% cysteine or 0.1% (W/V) α -2macroglobulin. The mixture is frozen and freeze dried at -65°C. at a vacuum of less than 50 millitorr or air dried at room temperature.

EXAMPLE 14

In Vitro Enzymatic Degradation

One cm.^{2} of each of the products of the above Examples is placed in 2.0 ml of 10 mM Tris-HCl pH 7.4 containing 25 mM calcium chloride and 100 units of type IV collagenase from Clostridium histolyticum (Sigma Chemical Co.) is added per mg of sample. The samples are placed in a 37°C. environment and the intactness of each sample is visually checked every 10 minutes. The time is recorded when each sample has visibly degraded into pieces smaller than about 0.5 um. The results are presented in Table I.

TABLE I
Physicochemical Properties of Collagen-Based Carriers

Carrier Composition	Crosslinking Treatment	Physical Form	r	C.R.T. (min)	E_f	HMS q mm
Type I Collagen	air dried	Sheet	22.2	72	0.37	508
Type I Collagen	SD(1)	Sheet	7.00	123	0.25	850
Type I Collagen	SD(2)	Sheet	4.11	213	0.19	1058
Type I Collagen	SD(3)	Sheet	4.20	380	0.22	1468
Type I Collagen	SD(5)	Sheet	3.13	460	0.22	1048
Type I Collagen	C(1)	Sheet	5.88	105	0.42	662
Type I Collagen	C(2)	Sheet	4.48	123	0.24	869
Type I Collagen	C(3)	Sheet	4.69	185	0.30	990
Type I Collagen	C(4)	Sheet	4.29	237	0.35	855
Type I Collagen	C(1)+SD(1)	Sheet	2.75	720	0.15	1186
Type I Collagen	C(1)+SD(2)	Sheet	2.16	960	0.16	1441
Type I Collagen	C(1)+SD(3)	Sheet	2.62	1440	0.12	1872
Type I Collagen	SD(3)+C(1)	Sheet	2.75	960	0.14	1959
Type I Collagen	SD(3)+C(2)	Sheet	3.03	960	0.16	1833
Type I Collagen	SD(3)+C(3)	Sheet	3.06	960	0.11	1637
Type I Collagen + 3% HA/PG Complex	air dried	Sheet	23.3	68	0.34	615
Type I Collagen + 3% HA/PG Complex	SD(3)+C(1)	Sheet	2.31	1440	0.15	2275
Type I Collagen + 3% HA/PG Complex	C(1)+SD(3)	Sheet	2.56	1440	0.13	3434
Type I Collagen	P	Sheet	3.47	1515		
Type I Collagen	P+SD(3)	Sheet	1.72	3180		

Abbreviations:

r = swelling ratio

C.R.T. = collagenase resistance time

SD = severe dehydration at 110°C

( ) = duration of crosslinking in days

C = 1% cyanamide immersion at 22°C

HA/PG hyaluronate-proteoglycan complex (Sigma Chemical Co.)

E_f = strain at failure

HSM = high strain modulus

P = crosslinked using succinyl ester method

EXAMPLE 15

In Vitro Determination of Swelling Ratio

The swelling ratio of denatured collagen is inversely related to the degree of crosslinking. The products of the above Examples were boiled for 2 minutes in distilled water and then blotted between two napkins. A 1 kg. weight was placed on top of the napkins containing the materials for 20 seconds. The wet sample weight was recorded and the sample was then dried at 110°C. for 3 hours. After drying the weight was again recorded and the swelling ratio (r) was calculated from the following relationship:

$$r = 1/V_f$$

where

$$V_f = \frac{DW/P_c}{\frac{DW}{(P_c)} + \frac{(WW - DW)}{(P_{H20})}}$$

; and where

DW and WW are the dry and wet weights, P_c and P_{H20} are the material and water densities, respectively. The results are presented in the aforementioned Table I.

EXAMPLE 16

Mechanical Properties of Collagen-Based Matrices

Sponges and sheets prepared in accordance with the above Examples were cut into rectangular (4.0 cm x 1.0 cm) strips and immersed in phosphate buffer solution pH 7.5 for 20 minutes prior to mechanical testing. The strips were tested in uniaxial tension at 22°C. at a strain rate of 10%/minute using an Instron Model 1122 testing device. The ends of the strips were placed in pneumatic grips that were closed under a pressure of 40 psig. with a gage length of 20 mm. Stress-strain curves were obtained from which the Young's moduli at high (HSM) and low strains were calculated. The strain at which the

low modulus region ended was designated e_L and the strain at failure was denoted e_f. The strain results are presented in Table I above.

EXAMPLE 17

Subcutaneous Biocompatibility of Collagen-Based Matrix

Sponges and sheets prepared in accordance with to the above Examples were tested for biocompatibility subcutaneously after sterilization by exposure 2.5 M rads of gamma radiation. Implantation was carried out under sterile conditions using 350 gr. white female guinea pigs as test animals.

A 1 cm. cutaneous incision was made on one side of the back and the skin was separated from the fascial layer and a 1 cm. x 1 cm. piece of the implant was placed in this space. The edges of the skin were fastened together over the implant using wound clips.

Animals were sacrified on the 6th, 9th and 12th post implantation day and the tissue containing the carrier was placed in Carson's fixative and processed for histological studies. The results are presented in the following Table II.

TABLE II
In Vivo Biocompatibility of Collagen-Based Carrier Sponges Implanted Subcutaneously

Carrier Composition	Crosslinking Treatment	Duration (days) of Implantation	Comments
Type I Collagen	None	6	No observable inflammatory response, no ingrowth, implant intact
Type I Collagen	None	9	No observable inflammatory response, no ingrowth, implant intact
Type I Collagen	None	12	No observable inflammatory response, no ingrowth, implant intact
Type I Collagen	SD(3)	6	No observable inflammatory response, no ingrowth, implant intact
Type I Collagen	C(1)+SD(3)	6	No observable inflammatory response, some ingrowth at edges of sponge, implant intact
Type I Collagen	C(1)+SD(3)	9	No observable inflammatory response, peripheral ingrowth, implant intact
Type I Collagen	P	6	No observable inflammatory response, no ingrowth, implant intact
Type I Collagen	P+SD(3)	6	No observable inflammatory response, good ingrowth on periphery, implant intact

Abbreviations:

P = crosslinked using succinyl ester method
SD = severe dehydration

EXAMPLE 18

Preparation of Diffusion Control Layer on a Matrix Layer

Using sponges and sheets prepared in accordance with to Examples 4 to 10, a 1 mm. layer of Silastic Medical Grade adhesive was applied to the surface of the matrix layer using a spatula. The diffusion control layer is cured by application of a vacuum of 100 millitorr for a period of 2 hours at 22°C. The resultant complex of the diffusion control and matrix layers was placed in contact with full thickness dermal wounds.

EXAMPLE 19

Biocompatibility of Diffusion Control and Matrix Layers on Full Thickness Dermal Wounds

Sponges and sheets prepared according to Example 18 were tested after radiation sterilization as dressings on open dermal wounds on female Hartley Albino 350 gr. white guinea pigs. Each animal was separately fed and weighed for 4 days prior to testing. One day before testing, the animal was shaved using an electric clipper followed by depilatory treatment with Nair and washed. The animal was then anesthetized by exposure to ether and its back washed with providone-iodine and alcohol solutions. A 2 cm. x 2 cm. piece of material composed of matrix and diffusion control layers soaked in a phosphate buffer solution (0.182 M phosphate and 0.154 M NaCl), was placed on a full thickness dermal wound on the same area with the matrix layer against the panniculus carnosus. The matrix and the diffusion control layers were sutured to the wound bed at the edges of the dressing using chrome tanned gut sutures. The animal was bandaged by placing sterile cotton dressing sponge over the wound dressing and then wrapped with an Elasticon elastic tape (Johnson and Johnson Products, Inc.) secured around the neck. Animals were housed three to a cage during the experiment.

The animals were monitored daily and their bandages examined for tears. Animals were sacrified at 6, 9 and 12 day post

implantation and the implant and wound beds were excised, fixed and processed for histological examination. The results are presented in Table III.

TABLE III
In Vivo Biocompatibility of Silicone-Coated Collagen-Based Carriers Implanted on Full Thickness Excised Dermal Wounds

Carrier Composition	Crosslinking Treatment Method (duration in days)	Duration (days) of Implantation	Comments
Type I Collagen	SD(3)	6	N.I.R., I.I., fibroblast ingrowth, synthesis of granulation tissue below implant
Type I Collagen	SD(3)	9	N.I.R., I., fibroblast ingrowth, remodeling of granulation tissue within and below implant
Type I Collagen	SD(3)	12	N.I.R., I.I., extensive fibroblast ingrowth, remodeling of granulation tissue within and below implant
Type I Collagen	SD(3)C(1)	6	N.I.R., I.I., slight fibroblast ingrowth, of granulation tissue formation below implant
Type I Collagen	SD(3)C(1)	9	N.I.R., I.I., slight fibroblast ingrowth, extensive remodeling of granulation tissue below implant
Type I Collagen	SD(3)C(1)	12	N.I.R., I.I., slight fibroblast ingrowth, extensive remodeling of granulation tissue below implant
Type I Collagen	(1)SD(3)	6	N.I.R., I.I., slight fibroblast ingrowth, remodeling of granulation tissue below implant

Type I Collagen	C(1)SD(3)	9	N.I.R., I.I., extensive fibroblast ingrowth, granulation tissue within and below implant
Type I Collagen	P	6	N.I.R., I.I., slight fibroblast ingrowth, granulation tissue below implant
Type I Collagen + 1% Laminim	SD(3)	6	W.I.R., I.I., slight fibroblast and capillary ingrowth, granulation tissue within and below implant
Type I Collagen + 5% Laminim	SD(3)+C(1)	6	W.I.R., I.I., extensive capillary ingrowth, granulation tissue within and below implant
Type I Collagen + 5% HA/PG	SD(3)+C(1)	6	N.I.R., I.I., extensive fibroblast ingrowth, granulation tissue within and below implant
Type I Collagen + 5% HA/PG	SD(3)+C(1)	9	N.I.R., I.I., extensive fibroblast ingrowth, remodeled granulation tissue below implant
Type I Collagen + 5% HA/PG	SD(3)+C(1)	12	N.I.R., I.I., implant remodeled after extensive fibroblast ingrowth and migration of epidermis
Type I Collagen + 1% Fibronectin +1% HA/PG	C(1)+SD(3)	9	N.I.R., I.I., complete fibroblast ingrowth into sponges remodeled granulation tissue below sponge
Type I Collagen + 1% Fibronectin +1% HA/PG	C(1)+SD(3)	12	N.I.R., I.I., implant remodeled after extensive ingrowth and migration of epidermis

Type I Collagen + 1% Fibronectin +1% HA/PG	C(1)+SD(3)	12	N.I.R., I.I., implant remodeled after extensive ingrowth and migration of epidermis
Type I Collagen + 1% Fibronectin	P	6	N.I.R., I.I., extensive fibroblast ingrowth, granulation tissue within implant
Type I Collagen + 1% Fibronectin	P	9	N.I.R., I.I., extensive fibroblast ingrowth, granulation tissue within implant
Type I Collagen + 1% Fibronectin	P	12	N.I.R., I.I., partial epidermal migration below implant
Type I Collagen + 1% Fibronectin	P+SD(3)	12	N.I.R., I.I., extensive fibroblast ingrowth and remodeling of granulation tissue

Abbreviations:

N.I.R. = no inflammatory response

W.I.R. = weak inflammatory response

SD = severe dehydration

C = cyanamide treatment

I.I. = implant intact

P = crosslinked by succinyl ester formation

( ) = duration of crosslinking in days

HA/PG = hyaluronate-proteoglycan complex

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, within the scope of the appended claims, the inventions may be practised otherwise than as particularly described.

What is Claimed:

1. A method of preparing a novel collagen-based matrix which comprises:

a.) admixing with a liquid medium a collagen selected from the group consisting of types I, II and III collagen to form a collagen-based substrate;

b.) introducing into the product of step a.) a crosslinking agent selected from the group consisting of a carbodiimide and a bifunctional succinimidyl active ester; and

c.) subjecting said product of step b.) to elevated temperatures under a vacuum to produce said collagen-based matrix.

2. The methods as defined in claim 1 wherein step c.) is performed prior to step b.).

3. The method as defined in Claims 1 or 2 wherein said liquid medium is a solvent for said collagen.

4. The method as defined in Claims 1 or 2 wherein said liquid medium is a dispersing agent for said collagen.

5. The method as defined in Claims 1 or 2 wherein said crosslinking agent is said carbodiimide and is in solution at a concentration of from about 0.1 to 10% (W/V).

6. The method as defined in Claim 5 wherein said carbodiimide solution and collagen-based substrate are maintain at a temperture of from 2 to 40°C. for a period of 2 to 96 hours prior to further processing.

7. The method as defined in Claim 6 wherein said carbodiimide solution is at a pH of from 2 to 11.

8. The method as defined in Claims 1 or 2 wherein said crosslinking agent is said bifunctional succinimidyl active ester and is in solution at a concentration of from about 0.1 to about 15.0% (W/V).

9. The method as defined in Claim 8 wherein said bifunctional succinimidyl active ester solution and said collagen-based substrate are maintained at a temperature of from 2 to 40°C. for a period of 2 to 96 hours prior to further processing.

10. The method as defined in Claims 1 or 2 where excess crosslinking agent is removed prior to step c.).

11. The method as defined in Claims 1 or 2 wherein step c.) is effect at a temperature of from 50°C. to about 200°C. at a vacuum of less than 50 millitorr.

12. The method as defined in Claim 11 wherein step c.) is effected for a period of time of from 2 to 96 hours.

13. The method as defined in Claims 1 or 2 wherein said collagen-based substrate is subjected to freeze drying prior to step b.) in said method of forming a collagen-based matrix in sponge form.

14. The method as defined in Claim 13 wherein said collagen-based substrate is solidified prior to the step of freeze drying.

15. The method as defined in Claim 14 wherein the step of freeze drying is effected under a vacuum of less than about 50 millitorr and at a temperature of from about 22°C. to -100°C.

16. The method as defined in Claim 15 wherein said collagen-based substrate is solidified at a temperature of from 0°C. to -100°C.

17. The method as defined in Claim 13 wherein said intermediate collagen-based matrix is subjected to freeze drying prior to step c.).

18. The method as defined in Claim 17 wherein said intermediate collagen-based matrix is solidified prior to freeze drying prior to step c.).

19. The method as defined in Claim 17 wherein the step of freeze drying is effected under a vacuum of less than about 50 millitorr and at a temperature of from about 22°C. to -100°C.

20. The method as defined in Claim 17 wherein said collagen-based substrate is solidified at a temperature of from 0°C. to -100°C.

21. The method as defined in Claims 1 or 2 wherein said collagen-based substrate is dried prior to step b.) in said method of forming a collagen-based matrix in sheet form.

22. The method as defined in Claim 21 wherein drying is effected at a temperature of from 4°C. to 40°C. for a time of from 2 to 96 hours.

23. The method as defined in Claims 1 or 2 wherein a carrier compound is introduced into said collagen-based matrix during formation of said collagen-based matrix, said carrier compound being selected from the group consisting of type IV collagen, type V collagen, fibronectin, laminin, hyaluronate, proteoglycan, epidermal growth factor, platelet derived growth factor, angiogenesis factor, antibiotic, antifungal agent, spermacidal agent, enzyme and enzyme inhibitors.

24. The method as defined in Claim 23 wherein said collagen based substrate is subjected to freeze drying prior to step b.) in said method of forming a collagen-based matrix in sponge form.

25. The method as defined in Claim 24 wherein said collagen-based substrate is solidified prior to the step of freeze drying.

26. The method as defined in Claim 25 wherein the step of freeze drying is effected under a vacuum of less than about 50 millitorr and at a temperature of from about 22°C. to -100°C.

27. The method as defined in Claim 26 wherein said collagen-based substrate is solidified at a temperature of from 0°C. to -100°C.

28. The method as defined in Claim 24 wherein said intermediate collagen-based matrix is subjected to freeze drying prior to step c.).

29. The method as defined in Claim 28 wherein said intermediate collagen-based matrix is solidified prior to freeze drying prior to step c.).

30. The method as defined in Claim 28 wherein the step of freeze drying is effected under a vacuum of less than about 50 millitorr and at a temperature of from about 22°C. to -100°C.

31. The method as defined in Claim 28 wherein said collagen-based substrate is solidified at a temperature of from 0°C. to -100°C.

32. The method as defined in Claim 23 wherein said collagen-based substrate is dried prior to step b.) in said method of forming a collagen-based matrix in sheet form.

33. The method as defined in Claim 32 wherein drying is effected at a temperature of from 4°C. to 40°C. for a time of from 2 to 96 hours.

34. The method as defined in Claim 33 and further including the step of overlaying said collagen-based matrix in sheet form with a diffusion layer.

35. The method as defined in Claim 34 wherein said diffusion layer is nonporous.

36. The method as defined in Claim 34 wherein said nonporous diffusion layer is biodegradable.

37. The method as defined in Claim 34 wherein said diffusion layer is formed of a non-biodegradable polymer.

38. A collagen-based matrix formed of a collagen selected from the group consisting of type I, II and III collagen of a molecular weight of at least 1.0×10^6.

39. The collagen-based matrix as defined in Claim 38 wherein said molecular weight is in excess of 50×10^6.

40. The collagen-based matrix as defined in Claim 39 basing a molecular weight between cross-links of from 1,000 to 100,000 via covalent bonding.

41. The collagen-based matrix as defined in Claim 38 having interstices of a pore size of from 3 to 100 μm.

42. The collagen-based matrix as defined in Claim 38 and further including a carrier compound selected from a group consisting of type IV collagen, type V collagen, fibronectin, laminin, hyaluronate, proteoglycan, epidermal growth factor, platelet derived growth factor, angiogenesis factor, antibiotic, antifungal agent, spermacidal agent, enzyme and enzyme inhibitor.

43. The collagen-based matrix as defined in Claims 38 or 42 in sheet form.

44. The collagen-based matrix as defined in Claim 43 and further including a diffusion layer.

45. The collagen-based matrix as defined in Claim 44 and further including a nonporous diffusion layer applied to either side of said collagen-based matrix.

46. The collagen-based matrix as defined in Claim 45 wherein said nonporous diffusions layer are biodegradable.

47. The collagen-based matrix as defined in Claim 44 wherein said diffusion layer is adhered tightly to said collagen-based matrix.

48. The collagen-based matrix as defined in Claim 47 wherein said collagen-based matrix and said diffusion layer have a combined tensile strength of at least 100 psi.

49. The collagen-based matrix as defined in Claim 47 wherein said collagen-based matrix and said diffusion layer have a deformation of at least 10%.

50. The collagen-based matrix as defined in Claim 43 having a swelling ratio of at least about 2.5.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 85/00504

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 08 L 89/06; A 61 L 15/04; A 61 K 9/22

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 08 L A 61 L; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	DE, A, 2734503 (FA. CARL FREUDENBERG) 1 February 1979, see claims 1,5, and 6, page 9, paragraph 3 and page 10, paragraph 2	1-50
Y	US, A, 3098693 (JOHN C. SEEHAN) 23 July 1963, see column 3, lines 50-74; examples 17, 41 and 50 and claim 14	1-50
A	Chemical Abstracts, volume 82, nr. 12, 24 March 1975, CHONAN Yasumasa et al.:"Chemical modification of collagen fibers with carbodiimides" seepage 80, abstract 74485b, & Hikaku Kagaku, 1974, 20(1) 29-36,	
A	US, A, 4412947 (GHEORGHE CIOCA) 1 November 1983	
A	The Journal of Clinical Pharmacology, August-September 1973, Albert L. Rubin et al.: "Collagen as a Vehicle for drug delivery"	

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th July 1985	20 AOUT 1985
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see pages 309-312	
Y	US, A, 3300470 (HARLAND H. YOUNG) 24 January 1967, see column 3, lines 21-30	1-50
A	Chemical Abstracts, volume 80,nr.22, 3 June 1974, Mary Jo White et al.: " Collagen films" see page 260, abstract 124729r & Biomater, Med. Devices Actif. Organs, 1973, 1(4) 703-15	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/US 85/00504 (SA 9275)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 23/07/85

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
DE-A- 2734503	01/02/79	None		
US-A- 3098693		None		
US-A- 4412947	01/11/83	FR-A-	2527621	02/12/83
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